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Substantivity of Chlorhexidine to Human Dentin

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Abstract

Objectives—To better comprehend the role of CHX in the preservation of resin-dentin bonds, this study investigated the substantivity of CHX to human dentin.

Material and Methods—Dentin disks (n= 45) were obtained from the mid-coronal portion of human third molars. One-third of dentin disks were kept mineralized (MD), while the other two-thirds had one of the surfaces partially demineralized with 37% phosphoric acid for 15 s (PDD) or they were totally demineralized with 10% phosphoric acid (TDD). Disks of hydroxyapatite (HA) were also prepared. Specimens were treated with: 1) 10 µL of distilled water (controls), 2) 10 µL of 0.2% chlorhexidine diacetate (0.2% CHX) or 3) 10 µL of 2% chlorhexidine diacetate (2% CHX). Then, they were incubated in 1 mL of PBS (pH 7.4, 37 °C). Substantivity was evaluated as a function of the CHX-applied dose after: 0.5h, 1h, 3h, 6h, 24h, 168h (1wk), 672h (4wks) and 1344h (8wks) of incubation. CHX concentration in eluates was spectrophotometrically analyzed at 260 nm.

Results—Significant amounts of CHX remained retained in dentin substrates (MD, PPD or TDD), independent on the CHX-applied dose or time of incubation (p<0.05). High amounts of retained CHX onto HA were observed only for specimens treated with the highest concentration of CHX (2%) (p<0.05).

Conclusion—The outstanding substantivity of CHX to dentin and its reported effect on the inhibition of dentinal proteases may explain why CHX can prolong the durability of resin-dentin bonds.

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Keywords

Dentin; Chlorhexidine; Binding; Substantivity

Introduction

Chlorhexidine (1,1'-hexamethylene-bis-5(4-chlorophenyl)bisguanide CHX, figure 1) is a symmetric molecule with two ionizable guanidine moieties. Its pKa values are 2.2 and 10.3, thereby making it cationic over the entire range of physiological pH values [1]. CHX has been shown to be effective against various oral bacteria. Due to its broad antimicrobial spectrum (i.e. against gram positive/negative bacteria and fungi), CHX has been used to adjunctively treat either endodontic [2,3] or periodontal diseases [4,5] and to arrest/prevent caries progression [6,7].

The recent finding that CHX also has potent anti-MMP-2, -8 and -9 activity [8] encouraged some researchers to determine whether CHX could stabilize the organic matrix of resin-dentin bonds. This led to numerous *in vitro* [9-12] and *in vivo* studies [13-16] that demonstrated that CHX has beneficial effects on the preservation of resin-dentin bonds, thereby offering a valuable alternative to clinicians who seek to delay the degradation process of adhesive restorations.

The effectiveness of CHX, as an antimicrobial or an antiproteolytic agent, has been reported to be related with its substantivity to oral/dental structures [17-19]. Substantivity is the prolonged association between a material (e.g. CHX) and a substrate (e.g. oral mucosa, oral proteins, dental plaque, dental surface), an association that can be greater and more extended than would be expected from a simple deposition mechanism. It is considered that the delivery of an agent to its site of action, in a biologically active form, and in effective doses, increases this agent effects for prolonged periods of time [20].

Substantivity of CHX, or its ability to be retained in dentin matrices, could be the reason why CHX-treated acid-etched dentin may form hybrid layers that are more stable over time [9-16]. The success of CHX in increasing the durability of resin-dentin bonds requires that more efforts be made toward understanding the mechanisms responsible for CHX binding to mineralized and demineralized dentin, in an attempt to optimize how CHX should be used clinically to maximize its retention and effectiveness.

Studies on substantivity of CHX to oral structures began in the early seventies [21,22]. Most of these studies investigated the retention of CHX in oral surfaces in order to determine its capacity to inhibit or decrease the bacterial growth/activity. Accordingly, as long as the number and growth of bacteria were controlled, CHX would be considered retained and effective. However, prolonged inhibition of bacteria growth/activity is merely an indirect way to estimate the substantivity of CHX to oral structures, and it may not be appropriate to explain its long-term antiproteolytic function.

The purpose of this study was to investigate the *in vitro* substantivity of CHX to human mineralized *versus* demineralized dentin. The tested null hypotheses were that: 1) CHX substantivity to mineralized and demineralized dentin is not different; 2) CHX substantivity to dentin substrates is independent on the concentration of applied CHX.

Material & Methods

Chlorhexidine diacetate monohydrate salt (CHX) was purchased from Fluka (St Louis, MO, USA). Reagent grade hydroxyapatite and other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) if not otherwise specified.

Specimen Preparation

Forty-five extracted non-carious human third molars were collected from young patients (20 – 28 years old) after their informed consent had been obtained under a protocol reviewed and approved by the Human Assurance Committee of the University of São Paulo, São Paulo, Brazil. Teeth were stored in 0.9% NaCl containing 0.02% sodium azide at 4 °C for less than one month. After organic debris/calculus removal, teeth were sectioned at the cementum-enamel junction to remove the roots. The pulp tissue was scraped off with sterile spoon excavators. Enamel and cementum were completely removed from the crown segment with high-speed diamond burs under copious water cooling. Dentin disks (0.5 ± 0.02 mm thick and 6.0 ± 0.01 mm diameter) were obtained from the mid-coronal portion of each tooth using a slow speed diamond saw (Labcut 1010, Extex Corp, Enfield, CT, USA) under water cooling followed by the use of a diamond-incrusted coring drill with a 6 mm internal diameter (Continental Diamond Tool Corp., New Haven, IN, USA). Dentin disks were ultra-sonicated in distilled-water bath for 15 minutes. Then, they were pre-dried in a sealed desiccator containing fresh silica gel at 37 °C, until a constant dry mass was obtained in analytical balance (Mettler Toledo Inc., Columbus, OH, USA) (i.e. 0.057 ± 0.009 g). Afterwards, they were individually immersed in distilled water at 37 °C for 1 week, gently wiped with absorbent paper and weighed in an analytical balance for determination of their wet mass (i.e. 0.062 ± 0.01 g). The inner, exposed surface of dentin disks (i.e. those that previously were in close contact with the pulp chamber) was completely covered with two layers of a nail varnish (Risqué, Niasi S/A, São Paulo, SP, Brazil), left to dry for 30 minutes (at 25 °C, 56% relative humidity) and had their dry mass re-measured (i.e. 0.063 ± 0.008 g).

Specimens were assigned for one of the following groups (n= 15): 1) dentin disks were kept mineralized (MD); 2) the outer, uncovered dentin surface of dentin disks was partially demineralized with liquid 37% phosphoric acid for 15 s and thoroughly rinsed with distilled water for 60 s (PDD); 3) dentin disks were totally demineralized with liquid 10% phosphoric acid for 12 hours and washed for 24 hours (TDD). Although a pilot study showed that the selected nail varnish is apparently acid-resistant (data not shown), care was taken to avoid direct contact between the varnish-covered dentin surface and the phosphoric acid solution. Disks of synthetic hydroxyapatite were also prepared [23]. One of the surfaces of these disks was completely covered with two layers of the same nail varnish while the other was kept exposed, as previously described for the dentin disks. Additionally, films of nail varnish (0.5 ± 0.01 mm thick and 6.0 ± 0.01 mm diameter) were also prepared.

Treatment of specimen surface

The exposed, dried, uncovered surface of each substrate (i.e. MD, PDD, TDD and HA) was treated with: 1) 10 µL of distilled water (controls); 2) 10 µL of 0.2% CHX (3.2 mM) or 3) 10 µL of 2% CHX (32.0 mM). The test solutions were applied uniformly on the specimens' surface using a micro-syringe and they were left undisturbed for 30 s. No excess solution was removed from the specimens' surface. With a pair of small forceps, the specimens were carefully transferred to 2-mL plastic centrifuge tubes containing 1 mL of PBS (pH 6.8) and incubated at 37 °C. Film-like specimens of nail varnish were also individually incubated under same conditions.

Analysis of Eluates

The 1 mL PBS contents of each tube containing any eluted CHX were spectrophotometrically analyzed in quartz cuvettes at 260 nm [18] after the following periods of incubation: 0.5 hour, 1 hour, 3, 6, 24, 168, 672 and 1344 hours. The eluates were never discarded so that after measuring their absorbance values, at each determined period, the eluates were returned to the same plastic centrifuge tubes for additional incubation of specimens. The cumulative concentration of CHX in the eluates was estimated as a function of their UV-absorbances plotted against a CHX standard curve. Substantivity of CHX to tested substrates was expressed (in percentage) based on the CHX-applied dose. In theory, if CHX was not able to bind to the tested substrates, one should expect to detect the entire amount of applied CHX (10 μ L of 0.2% or 2%) to be released into 1000 μ L of PBS. This theoretical amount is 32 nmoles (in 1000 μ L) for specimens treated with 0.2% CHX and 320 nmoles (in 1000 μ L) for specimens treated with 2% CHX.

Data were analyzed by a three-way repeated measures ANOVA with substrate (MD, PDD, TDD and HA), specimen treatment mode (without, with 0.2% CHX or with 2% CHX) and time of incubation (0.5 to 1344 h) as main factors, and with the eluate as factor of repetition. Post hoc multiple comparisons were performed using Holm-Sidak test. Statistical significance was preset at $\alpha=0.05$.

Results

UV-absorbances (A) for control specimens (i.e. not treated with CHX) were low (0.2 ± 0.05 A) and significantly different from the CHX-treated specimens, regardless of the tested substrate, CHX concentration or time of incubation ($p<0.05$) (data not shown). Such low values were coincident with the mean UV-absorbance (0.2 ± 0.1 A) of film-like specimens of pure varnish (data not shown), indicating that the low positive UV-absorbances of control groups were likely due the presence of leaching species from the varnish-covered surface. To confirm it, specimens of each substrate were prepared and stored in PBS without being covered with varnish and treated with CHX. As expected, these specimens exhibited null or negative UV-absorbance (data not shown). Thus, since all experimental groups had their own control (i.e. specimens that were covered with nail varnish and were not treated with CHX), the mean UV-absorbance of each control group was subtracted from the mean UV-absorbance of the corresponding CHX-treated group.

Substantivity of CHX for all treated substrates expressed as a percentage the CHX-applied dose is summarized in Table 1, while Figure 2 shows graphically the percent of bound CHX, separately, to specimens treated with 0.2% (Figure 2a) and 2.0% CHX (Figure 2b).

For substrates treated with 0.2% CHX, the lowest substantivity was exhibited for HA specimens, while the highest substantivity was observed for PDD specimens, regardless of the time of incubation. Statistically significant differences, independently on the incubation time, were found between HA and the other three tested substrates (MD, PPD and TDD) ($p<0.05$; Table 1 and Figure 2a). For all periods of incubation, significant differences were also observed between MD and TDD substantivities, with MD > TDD ($p<0.05$); and between PDD and TDD substantivities, with PDD > TDD ($p<0.05$) (Table 1 and Figure 2a). At the final period of incubation (1344 h), only 3% of 0.2%-applied CHX was bound to HA specimens while over 97% of the applied CHX remained bound to PDD, 85% to MD and 74% to TDD specimens. Differences in the substantivity over incubation time for specimens were significant only for PDD substrate (Table 1) with the longest time periods (from 168 hours on) ($p<0.05$) exhibiting higher substantivity than the earliest times (from 0.5 to 24 hours). Conversely, for HA substrate, the longest periods of incubation resulted in

significantly lower substantivity when compared to that measured at the earliest periods ($p < 0.05$).

For substrates treated with 2% CHX, the lowest substantivity was verified for HA and PDD specimens, while the highest substantivity was observed for TDD specimens. Statistically significant differences, regardless of the incubation time, were found between TDD and the three other substrates ($p < 0.05$) (Table 1 and Figure 2b). For all periods of incubation, significant differences were also found between MD and HA substrates, with MD > HA ($p < 0.05$); as well as between MD and PDD ($p < 0.05$), with MD > PDD (Table 1 and Figure 2b). At the final period of incubation, 49% of 2%-applied CHX was bound to HA specimens while over 83% of the applied CHX remained bound to TDD, 68% to MD and 54% to PDD specimens. Differences in the substantivity over the incubation period occurred only to PDD specimens, with the longest two time periods of incubation (672 and 1344 h) exhibiting values of substantivity higher than the earliest periods ($p < 0.05$) (Table 1).

The 0.2% CHX solution provided higher mean substantivity for all dentin substrates (MD, PDD and TDD) than did the 2% CHX solution ($p < 0.05$). In general, the eluate resulting from specimens treated with 2% CHX exhibited very high values of UV-absorbance (2.2 to 2.7A), indicating that a great amount of CHX was released when specimens were incubated in PBS, in contrast to the results obtained when samples were treated with 0.2% CHX (i.e. UV-absorbances from 0.9 to 1.3A) (data not shown). UV-absorbance values as high as 2.5 to 2.7 should be carefully considered since they are close to the photometric readout limit of the spectrophotometer (i.e. 3.0A).

Discussion

Many clinicians currently apply 2% CHX to acid-etched dentin during resin bonding in an attempt to increase the durability of resin-dentin bonds by inhibiting endogenous MMPs in the dentin matrix. The present protocol aimed to determine whether CHX may preferentially bind the inorganic or organic phase of dentin when it is applied, with no further rinsing, to acid-etched dentin right after the acid-etching step. A considerable amount of CHX remained retained to dentin substrates (MD, PPD or TDD), independent on the CHX-applied dose and time of incubation. For most of tested periods, the substantivity of CHX to mineralized and partially-demineralized dentin was similar when these substrates were treated with 0.2% CHX. Nevertheless, using this lower concentration of CHX (0.2%), mineralized and totally-demineralized dentin specimens exhibited different substantivity (Fig. 2a). The opposite ensued when 2% CHX was used; that is, mineralized and partially-demineralized dentin exhibited dissimilar patterns of CHX substantivity, while mineralized and totally-demineralized did not differ significantly (Fig. 2b). These results support the partial rejection of the first study null hypothesis. The results also showed that substantivity of CHX to dentin substrates varied according to the concentration of CHX solution, which then requires the acceptance of the second study null hypothesis.

When ionized in water [1], CHX is characterized by being a strong base with cationic properties. Its mechanism of action is that the cationic part of its molecule binds to the negatively-charged surface/site of target-substrates. Studies on the specific interaction of CHX with oral tissues are not conclusive, but is quite likely that it results from a cationic-anionic reaction, involving an electrostatic attraction between the protonated amine groups of CHX and the mineral phosphates [24,25] and/or phosphoproteins, carboxylic and hydroxyl groups of collagen and noncollagenous phosphoproteins (anionic reactants) [26,27].

Exploring the nature of the interaction of CHX with hydroxyapatite, Misra (1994) [25] studied the kinetics of CHX uptake onto different synthetic hydroxyapatites using a solvent (*p*-dioxane) in which CHX is soluble. With low CHX concentrations (2 to 5 mmol/L or approximately 0.2 to 0.45 %) there is no interaction with synthetic hydroxyapatites, while with higher concentrations (10 to 50 mmol/L or approximately 1.0 to 4.5%) CHX was progressively retained with time. After extensive washing with *p*-dioxane and drying, only hydroxyapatite that was treated with higher concentrations of CHX (1.0 to 4.5%) exhibited, in SEM analysis, distinct birefringent crystals with the same refractive index (i.e. 1.47 – 1.48) as that of a phosphate salt. The retention of CHX in hydroxyapatite seems thus to be dependent on the precipitation of sparingly soluble phosphate salts, involving phosphate ion belonging to hydroxyapatite. Thus, the nature of CHX interaction with hydroxyapatite could be partially reactive and not exclusively adsorptive. From such a perspective, it was considered that when applying low concentrations of CHX (e.g. <0.2%), the salt - product of its reaction with hydroxyapatite - is probably soluble and there is no uptake. As the concentration of CHX is increased (e.g. >0.2%), the uptake becomes dependent on the crystallization of the phosphate salt [25]. The study concluded that any treatment with phosphate-containing solution before rinsing with CHX should enhance the retention of CHX to hydroxyapatite as a phosphate salt. Although Misra (1994) [25] had used CHX digluconate instead of CHX diacetate (as in the present protocol), we believe that these findings offer a reasonable explanation on why the HA specimens in the present study exhibited such low percentage of CHX retention (i.e. 3- 10%) when they were treated with 0.2 % CHX vs 2% CHX and stored in PBS (Table 1, Fig. 2 a,b).

Pioneering studies on the interaction between CHX and dental structures [22,28] suggested that hydroxyapatite alone would be incapable of retaining the whole amount of CHX that is available during mouthrinse [22]. Significantly more CHX can be taken by hydroxyapatite covered with a pellicle of salivary glycoproteins in comparison with uncovered hydroxyapatite [22,29,30]. In previous studies, it was suggested that formation of CHX-proteins salts of low solubility could play an important role in the retention of CHX in the oral cavity [31]. More recently, CHX was shown to be notably bound to biochemically modified type I collagen that was covalently coating titanium implant devices [32]. Indeed, the magnitude of and abiding retention of CHX observed in the current study for partially-(PDD) and, especially, totally-demineralized dentin specimens (TDD) (Table 1) indicates that CHX can markedly interact with organic components of dentin matrix. Again, in this case, it is assumed that the nature of CHX-dentin matrix interaction is governed by electrostatic forces, wherein protonated CHX presumably reacts with negatively-charged molecules of dentin matrix, such as with certain anionic organic domains ($^{-}\text{COOH}$ and/ or ^{-}OH) of collagen or even with anionic moieties of glycosaminoglycans [33] that in turn are closely related to collagen fibrils [34,35].

Results of the present study indicates that applying CHX to partially demineralized dentin (as it is used clinically) gives the opportunity for CHX to bind to both the collagen matrix and the underlying mineralized matrix. That may be why the percentage substantivity of 0.2% CHX to PDD was higher than either MD or TDD. Nevertheless, from the current findings it is also possible that while the substantivity of CHX to dentin may be co-supported by its interaction with hydroxyapatite, a stronger and more sustained reaction of CHX with organic components of dentin matrix seems to be preferential (Table 1). In fact, binding of CHX to dentin matrix components is probably the best way for CHX to inhibit collagen-bound proteases, exerting its antiproteolytic function in order to prolong the life-span of adhesive bonding restorations. It is likely that under clinical conditions the retention of CHX to dentin matrix can be enhanced by applying CHX to acid-etched dentin without rinsing, and then covering it with adhesive, thereby “sandwiching” the bound CHX between the underlying mineralized dentin and the polymerized adhesive restoration. This may

explain why *in vivo* CHX-treated hybrid layers exhibited none or few traces of deterioration over prolonged oral function [13-16].

Previous studies on the retentivity of CHX to dental tissues generally report periods of substantivity that are shorter than those observed in the present study. Most of those studies were interested in determining the minimal concentration for efficient microbial control [2,18,36,37]. However, these studies did not quantify the amount of CHX applied onto experimental specimens, so it is not technically possible to know whether all CHX was released. In the present study, the substantivity of CHX to dentin substrates was determined based on the CHX-applied dose. So, direct comparison between the previous reported values of CHX substantivity and those obtained in the current study would not be realistic.

It is interesting to note that increases of CHX concentration (i.e. from 0.2 to 2%) could either increase or decrease the magnitude of the interaction of CHX with dentin substrates. Even if after 56 days (i.e. 1344 hours) of incubation, the specimens of MD and PDD exhibited relatively high percentage of bound CHX (i.e. 68% and 54%, respectively) when they were treated with 2% CHX. These percentage values were significantly lower than those obtained when the same specimens were treated with 0.2% CHX (i.e. 86% and 97%, respectively) (Table 1). One of the models (Langmuir model) that describes chemical adsorption of substances onto specific sites within an adsorbent assumes that, due to repulsive intermolecular forces, once an adsorbate molecule (e.g. CHX) occupies a site on the outer surface of the adsorbent (e.g. dentin substrates) no further uptake can take place at that site, supporting the existence of a monolayer coverage [27]. Perhaps, application of the lower concentrations of CHX (0.2%) provided the formation of a relatively stable monolayer of retained CHX on MD and PDD specimens, while the higher concentration might have given only an oversaturation of CHX with a rapid release of its excess.

Conversely, the slight decrease in the substantivity of CHX to TDD specimens when treated with 0.2% CHX (i.e. 73% retention compared to 86% when treated with 2% CHX) does not necessarily seem to affect its effectiveness as an antiproteolytic agent. In general, enzyme inhibition can be effectively reached even at the presence of very low concentrations of a synthetic inhibitor. CHX has been shown to directly inhibit MMP-2, -8 and -9 activities at extremely low concentrations (i.e. 0.02% for MMP-8; 0.002% for MMP-9 and 0.0001% for MMP-2) [8]. These are the same MMPs that have shown to be present in human dentin [38-40]. While future studies should determine the optimal concentration for CHX inhibition of the proteolytic activity in dentin, it is encouraging to know that by treating acid-etched dentin with 0.2% CHX provided formation of hybrid layers that are as stable as those formed when the acid-etched dentin was treated with 2% CHX [11].

It is evident that by expressing substantivity only as a percentage of the CHX-applied dose does not give the complete dimension of current results. As already mentioned, it is possible that application of high concentrations of CHX may cause only an oversaturation of the substrate with a rapid release of CHX excess. However, considering that part of CHX may be washed out from primed dentin during application of liquid comonomers for bonding, the clinical saturation of dentin with a more concentrated solution of CHX could be beneficial. In these circumstances, the desorbed CHX may be incorporated into resin adhesive before its polymerization, permitting this resin to serve as a depot for slow release of CHX [41-43]. This is, at least indirectly, supported by the increased percentage of bound CHX with prolonged (from one to eight weeks) incubation time with PDD samples, which are closest to the clinical reality in adhesive bonding. The finding indicates that at least partially demineralized dentin surfaces can be reloaded with CHX. Since the benefits of incorporating CHX in resin still wait for substantiation, the speculation that the higher (i.e. 2%) concentration of CHX may be advantageous does not invalidate the previous evidences that

lower concentrations of CHX are successful in preserving the stability of resin-dentin bonds at least over one year of an *in vitro* storage [11].

For optimal durability of resin-dentin bonds, preservation of both components of hybrid layers (resin and dentin matrix) should be attained. We anticipate that in CHX-treated cavities, the collagen-bound proteases would probably remain inhibited for as long as CHX remained bound to dentin matrix [14,19]. In conclusion, the present results indicate that the substantivity of CHX to dentin may play a paramount role in the inhibition of collagen-bound proteases and, consequently, in the stability of CHX-treated resin-bonded interfaces.

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References

1. Nerurkar MJ, Zentner GM, Howard Rytting J. Effect of chloride in the releasing of chlorhexidine salts from methyl methacrylate: 2-hydroxyethyl methacrylate copolymer reservoir devices. *J Control Release* 1995;33:357–363.
2. Siqueira JF Jr, Paiva SS, Rôças IN. Reduction in the cultivable bacterial populations in infected root canals by a chlorhexidine-based antimicrobial protocol. *J Endod* 2007;33:541–547. [PubMed: 17437868]
3. Vianna ME, Horz HP, Conrads G, Feres M, Gomes BP. Comparative analysis of endodontic pathogens using checkerboard hybridization in relation to culture. *Oral Microbiol Immunol* 2008;23:282–290. [PubMed: 18582327]
4. Cosyn J, Wyn I, De Rouck T, Sabzevar MM. Long-term clinical effects of a chlorhexidine varnish implemented treatment strategy for chronic periodontitis. *J Periodontol* 2006;77:406–415. [PubMed: 16512755]
5. Duarte FF, Lotufo RF, Pannuti CM. Local delivery of chlorhexidine gluconate in patients with aggressive periodontitis. *J Int Acad Periodontol* 2008;10:31–35. [PubMed: 18333598]
6. Garcia MB, Nör JE, Schneider LG, Bretz WA. A model for clinical evaluation of the effect of antimicrobial agents on carious dentin. *Am J Dent* 2001;14:119–122. [PubMed: 11572285]
7. de Amorim RG, Leal SC, Bezerra AC, de Amorim FP, de Toledo OA. Association of chlorhexidine and fluoride for plaque control and white spot lesion remineralization in primary dentition. *Int J Paediatr Dent* 2008;18:446–451. [PubMed: 18489576]
8. Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinases 2, 8 and 9 by chlorhexidine. *Clin Diagn Lab Immunol* 1999;6:437–439. [PubMed: 10225852]
9. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, Ito S. Collagen degradation by host-derived enzymes during aging. *J Dent Res* 2004;83:216–221. [PubMed: 14981122]
10. Carrilho MR, Carvalho RM, de Goes MF, di Hipolito V, Geraldini S, Tay FR, Pashley DH, Tjäderhane L. Chlorhexidine partially preserves long-term dentin bond strength in vitro. *J Dent Res* 2007a;86:90–94. [PubMed: 17189470]
11. Breschi L, Cammelli F, Visintini E, Mazzoni A, Vita F, Carrilho M, Cadenaro M, Foulger S, Tay FR, Pashley DH. Influence of chlorhexidine concentration on the durability of etch-and-rinse dentin bonds: A 12-month in vitro study. *J Adhes Dent* 2009;11:191–198. [PubMed: 19603582]
12. Stanislawczuk R, Amaral RC, Zander-Grande C, Gagler D, Reis A, Loguercio AD. Chlorhexidine-containing acid conditioner preserves the longevity of resin-dentin bonds. *Oper Dent* 2009;34:481–490. [PubMed: 19678455]
13. Hebling J, Pashley DH, Tjäderhane L, Tay FR. Chlorhexidine arrests subclinical degradation of dentin hybrid layers in vivo. *J Dent Res* 2005;84:741–746. [PubMed: 16040733]

14. Carrilho MR, Geraldini S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L, Reis AF, Hebling J, Mazzoni A, Breschi L, Pashley D. In vivo preservation of the hybrid layer by chlorhexidine. *J Dent Res* 2007b;86:529–533. [PubMed: 17525352]
15. Brackett WW, Tay FR, Brackett MG, Dib A, Sword RJ, Pashley DH. The effect of chlorhexidine on dentin hybrid layers in vivo. *Oper Dent* 2007;32:107–111. [PubMed: 17427817]
16. Brackett MG, Tay FR, Brackett WW, Dib A, Dipp FA, Mai S, Pashley DH. In vivo chlorhexidine stabilization of hybrid layers of an acetone-based dentin adhesive. *Oper Dent* 2009;34:379–383. [PubMed: 19678441]
17. Basrani B, Santos JM, Tjäderhane L, Grad H, Gorduysus O, Huang J, Lawrence HP, Friedman S. Substantive antimicrobial activity in chlorhexidine-treated human root dentin. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;94:240–245. [PubMed: 12221393]
18. Franco CF, Pataro AL, E Souza LC, Santos VR, Cortés ME, Sinisterra RD. In vitro effects of a chlorhexidine controlled delivery system. *Artif Organs* 2003;27:486–491. [PubMed: 12752214]
19. Carrilho MR, Tay FR, Donnelly AM, Agee KA, Tjäderhane L, Mazzoni A, Breschi L, Foulger S, Pashley DH. Host-derived loss of dentin matrix stiffness associated with solubilization of collagen. *J Biomed Mater Res B Appl Biomater* 2009;90:373–380. [PubMed: 19090493]
20. Greenstein G, Polson A. The role of local drug delivery in the management of periodontal diseases: a comprehensive review. *J Periodontol* 1998;69:507–520. [PubMed: 9623893]
21. Løe H, Schiøtt CR. The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. *J Periodontal Res* 1970;5:79–83. [PubMed: 4254172]
22. Rølla G, Løe H, Schiøtt CR. The affinity of chlorhexidine for hydroxyapatite and salivary mucins. *J Periodontal Res* 1970;5:90–95. [PubMed: 4254174]
23. Akao M, Aoki H, Kato K. Mechanical properties of sintered hydroxyapatite for prosthetic applications. *J Mater Sci* 1981;16:809–812.
24. Sodhi RN, Grad HA, Smith DC. Examination by X-ray photoelectron spectroscopy of the adsorption of chlorhexidine on hydroxyapatite. *J Dent Res* 1992;71:1493–1497. [PubMed: 1324262]
25. Misra DN. Interaction of chlorhexidine digluconate with and adsorption of chlorhexidine on hydroxyapatite. *J Biomed Mater Res* 1994;28:1375–1381. [PubMed: 7829568]
26. Greenstein G, Berman C, Jaffin R. Chlorhexidine. An adjunct to periodontal therapy. *J Periodontol* 1986;57:370–377. [PubMed: 3522851]
27. Blackburn RS, Harvey A, Kettle LL, Manian AP, Payne JD, Russell SJ. Sorption of chlorhexidine on cellulose: mechanism of binding and molecular recognition. *J Phys Chem B* 2007;111:8775–8784. [PubMed: 17602516]
28. Rølla G, Løe H, Schiøtt CR. Affinity of chlorhexidine gluconate to hydroxylapatite and to salivary mucins. *Caries Res* 1971;5(1):23.
29. Emilson CG, Ericson T, Heyden G, Magnusson BC. Uptake of chlorhexidine to hydroxyapatite. *J Periodontal Res Suppl* 1973;12:17–21. [PubMed: 4269594]
30. Jenkins S, Addy M, Wade W. The mechanism of action of chlorhexidine. A study of plaque growth on enamel inserts in vivo. *J Clin Periodontol* 1988;15:415–424. [PubMed: 3183067]
31. Hjeljord LG, Rølla G, Bonesvoll P. Chlorhexidine-protein interactions. *J Periodontal Res Suppl* 1973;12:11–6. [PubMed: 4269593]
32. Morra M, Cassinelli C, Cascardo G, Carpi A, Fini M, Giavaresi G, Giardino R. Adsorption of cationic antibacterial on collagen-coated titanium implant devices. *Biomed Pharmacother* 2004;58:418–422. [PubMed: 15464868]
33. Müller G, Kramer A. In vitro action of a combination of selected antimicrobial agents and chondroitin sulfate. *Chem Biol Interact* 2000;124:77–85. [PubMed: 10670820]
34. Goldberg M, Takagi M. Dentine proteoglycans: composition, ultrastructure and functions. *Histochem J* 1993;25:781–806. [PubMed: 7507908]
35. Breschi L, Gobbi P, Lopes M, Prati C, Falconi M, Teti G, Mazzotti G. Immunocytochemical analysis of dentin: a double-labeling technique. *J Biomed Mater Res A* 2003;1(67):11–17. [PubMed: 14517856]

36. Mohammadi Z, Shahriari S. Residual antibacterial activity of chlorhexidine and MTAD in human root dentin in vitro. *J Oral Sci* 2008;50:63–67. [PubMed: 18403886]
37. García-Caballero L, Carmona IT, González MC, Posse JL, Taboada JL, Dios PD. Evaluation of the substantivity in saliva of different forms of application of chlorhexidine. *Quintessence Int* 2009;40:141–144. [PubMed: 19169446]
38. Martín de las Heras S, Valenzuela A, Overall CM. The matrix metalloproteinase gelatinase A in human dentine. *Arch Oral Biol* 2000;45:757–765. [PubMed: 10869489]
39. Mazzoni A, Mannello F, Tay FR, Tonti GAM, Papa S, Mazzotti G, DiLenarda R, Pashley DH, Breschi L. Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin. *J Dent Res* 2007;86:436–440. [PubMed: 17452564]
40. Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T, Tjäderhane L. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Arch Oral Biol* 2007;52:121–127. [PubMed: 17045563]
41. Riggs PD, Braden M, Patel M. Chlorhexidine release from room temperature polymerising methacrylate systems. *Biomaterials* 2000;21:345–351. [PubMed: 10656315]
42. Anusavice KJ, Zhang NZ, Shen C. Controlled release of chlorhexidine from UDMA-TEGDMA resin. *J Dent Res* 2006;85:950–954. [PubMed: 16998139]
43. Hiraishi N, Yiu CKY, King NM, Tay FR, Pashley DH. Chlorhexidine release and water sorption characteristics of chlorhexidine-incorporated hydrophobic/hydrophilic resins. *Dent Mater* 2008;24:1391–1399. [PubMed: 18439668]

Figure 1.
Chemical structure of chlorhexidine.

Figure 2.

Percentage of bound CHX to specimens treated with 0.2% (A) CHX or treated with 2% CHX (B) as a function of time. The percentage bound is relative to the amount applied. That is, little CHX remained bound to hydroxyapatite (HA), while most of the CHX applied to the various dentins remained bound.

Table 1
Percentage of CHX remaining bound (i.e. substantivity) over time as a function of the CHX-applied dose.

Substantivity of Chlorhexidine									
	0.5h	1h	3h	6h	24h	168h (1 wk)	672h (4 wks)	1344h (8 wks)	
MD 0.2% CHX	86.3 ± 5.1 ^{aA}	87.3 ± 5.0 ^{aA}	85.6 ± 4.0 ^{abAB}	85.6 ± 3.7 ^{aA}	88.7 ± 4.1 ^{aAB}	86.3 ± 3.2 ^{aA}	86.2 ± 3.9 ^{aA}	86.9 ± 3.2 ^{aA}	
MD 2.0% CHX	68.9 ± 3.8 ^{aB}	68.8 ± 5.0 ^{aB}	68.8 ± 3.5 ^{aB}	68.7 ± 4.6 ^{aB}	68.6 ± 3.2 ^{aC}	68.2 ± 4.0 ^{aC}	68.3 ± 3.1 ^{aD}	68.2 ± 2.8 ^{aC}	
HA 0.2% CHX	7.9 ± 1.0 ^{aD}	7.5 ± 1.8 ^{aD}	7.4 ± 2.2 ^{abE}	6.8 ± 1.5 ^{aD}	9.9 ± 3.1 ^{aE}	6.6 ± 1.2 ^{abE}	3.7 ± 0.9 ^{bF}	3.1 ± 1.2 ^{bE}	
HA 2.0% CHX	48.3 ± 2.1 ^{aC}	49.1 ± 1.8 ^{aC}	48.0 ± 3.3 ^{aD}	48.3 ± 1.2 ^{aC}	49.5 ± 1.5 ^{aD}	49.4 ± 1.9 ^{aD}	50.5 ± 3.0 ^{aE}	49.0 ± 2.7 ^{aD}	
PDD 0.2% CHX	92.6 ± 3.3 ^{abA}	91.3 ± 3.1 ^{bA}	90.8 ± 3.6 ^{bA}	90.9 ± 2.5 ^{bA}	90.9 ± 1.5 ^{bA}	97.1 ± 2.0 ^{aA}	97.5 ± 1.6 ^{aA}	97.9 ± 3.1 ^{aA}	
PDD 2.0% CHX	45.7 ± 2.7 ^{bC}	44.6 ± 2.0 ^{bC}	46.3 ± 1.5 ^{bD}	48.9 ± 2.6 ^{abC}	47.6 ± 3.0 ^{bD}	49.9 ± 2.4 ^{abD}	55.2 ± 2.8 ^{aE}	54.1 ± 2.9 ^{aD}	
TDD 0.2% CHX	74.4 ± 3.3 ^{aB}	75.9 ± 2.1 ^{aB}	72.3 ± 1.9 ^{aC}	71.2 ± 2.8 ^{aB}	75.1 ± 3.0 ^{aC}	75.0 ± 2.5 ^{aC}	76.1 ± 3.0 ^{aC}	74.2 ± 2.7 ^{aC}	
TDD 2.0% CHX	85.8 ± 4.5 ^{aA}	85.4 ± 3.5 ^{aA}	83.0 ± 2.0 ^{aB}	84.2 ± 3.1 ^{aA}	84.8 ± 1.6 ^{aB}	83.6 ± 4.1 ^{aB}	83.6 ± 2.3 ^{aB}	83.0 ± 4.7 ^{aB}	